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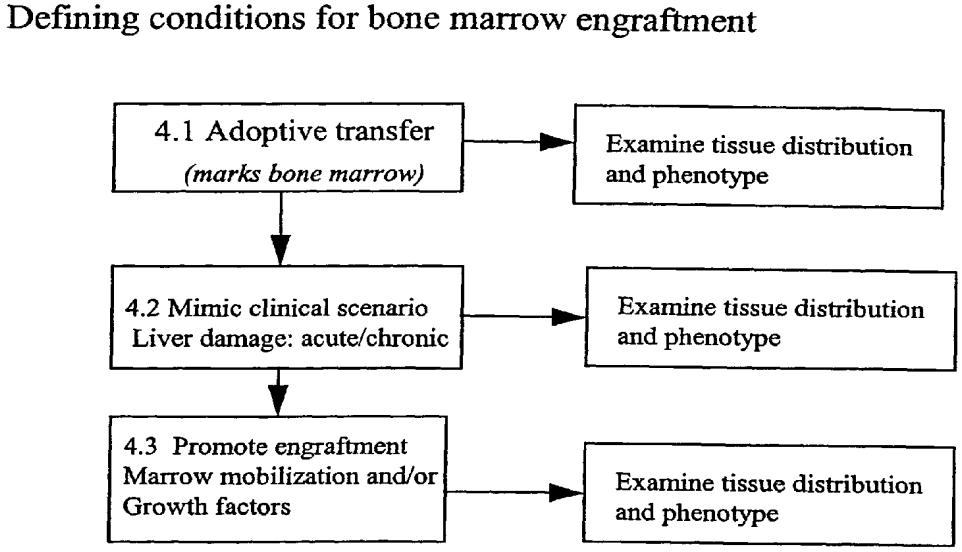
(54) Title: METHODS



(57) Abstract: A bone-marrow derived stem cell comprising a recombinant nucleic acid capable of expressing, in the bone-marrow derived stem cell or cell derived therefrom, a therapeutic product. The bone-narrow derived stem cell may be used in the treatment of tissue damage, for example liver damage, or in the correction of a genetic defect. The patient may be administered compositions which promote mobilisation of the stem cells and their maturation in to the desired type of differentiated cell. The bone-marrow derived stem cell may comprise a recombinant nucleic acid capable of expressing a cytotoxic product; this may be useful in removing the recombinant cells from the patient if the cells are no longer required.

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METHODS

This invention relates to methods for treating of tissue damage, particularly damage to the liver, and compositions useful therein.

Stem cells have the ability to divide for indefinite periods of time, and at the same time, give rise to specialized cells of the organs in which they are located. Cells that have the potential to produce the diverse cell types that make up the body might therefore provide replacements for tissues damaged by age, trauma and disease (1). Unlike blood stem cells, tissue stem cells have proved difficult to identify and isolate.

While it is widely appreciated that murine and human embryonic stem (ES) cells are highly malleable and can be coaxed to become many different cell types with therapeutic potential, the use of human ES cells raises a number of serious moral and ethical issues (2). However, the use of adult stem cells poses no such ethical quandary. Recent studies in mice and our studies in human tissue, suggest that stem cells normally resident in the bone marrow can differentiate into a wide variety of non-haemopoietic cell types and so may be as flexible in their options as ES cells (3-5).

Although the liver is normally mitotically quiescent in adulthood, substantial degrees of parenchymal loss can be restored through hepatocyte regeneration (6). Moreover, severe liver injury activates a potential stem cell compartment located in the intrahepatic biliary network that can also replace lost hepatocytes, existing in both experimental animals (7) and man (8). Nevertheless, patient death can still occur from acute and/or chronic hepatocyte loss resulting from drug or viral insult, indicating that this highly evolved

regenerative response can still fail in commonly occurring disease states. For example, worldwide over 300 million people are infected with Hepatitis B virus; failure to clear the virus results in hepatitis, fibrosis and eventually cirrhosis and liver cancer. The demonstration of the ability of murine bone marrow cells to transdifferentiate into skeletal muscle (3-5) raised the possibility that some cells in the bone marrow may be able to differentiate into other tissues, including hepatocytes, and that such cells may be able replace hepatocytes in regeneratively deficient scenarios.

Cross-sex and whole liver transplantation studies in rodents have indicated that bone marrow or extrahepatic stem cells can differentiate into hepatocytes (11,12). We have shown in the human liver that hepatocytes can also be derived from such extrahepatic sources (13). Following transplantation of male bone marrow we found Y chromosome-positive hepatocytes in the livers of female recipients. Similarly, we also found Y-positive hepatocytes in female livers that had been orthotopically transplanted into males, again indicative of an extrahepatic origin of these cells; these observations in humans have been confirmed by Theise et al. (14).

Hepatocytes derived from transplanted bone marrow have also been noted in a murine model of fatal hereditary type 1 tyrosinaemia (15), and here the most effective marrow-derived repopulating cells were identified by their antigenic profile, being c-kit^{high}Thy^{low}Lin^{-}Sca-1^{+}. These findings suggest that adult bone marrow stem cells, when displaced from their usual niche, are capable of being instructed to yield an epithelial lineage.

In the present invention, the bone marrow cells are used as vectors to carry recombinant therapeutic genes, for example to correct single gene defects,

particularly defects of liver synthesis (eg haemophilia) or to correct metabolic defects (eg diabetes mellitus), for example defects that can be corrected by expression in the liver, for example, factor VIII or IX insulin, or recombinant genes encoding products capable of modifying the immune or inflammatory responses of the body or promoting tissue repair and/or replacement/regeneration, for example antiviral cytokines. This allows patients to be treated using their own tissue and promotes formation of functional tissue.

A first aspect of the invention provides a bone-marrow derived stem cell comprising a recombinant nucleic acid capable of expressing, in the bone-marrow derived stem cell or cell derived therefrom, a therapeutic product.

The therapeutic product may be a nucleic acid or a polypeptide. By polypeptide is included glycoproteins, lipoproteins or other post-translationally modified proteins. Preferably the therapeutic product is a polypeptide.

The product may be capable of correcting a metabolic or genetic disorder, for example a disorder arising from a defect in a single gene. Thus, the product may be the product of an equivalent normal (non-defective) gene.

Alternatively, the product may be an antisense molecule that is capable of disrupting expression of a defective gene. This may be useful if the defective gene product has a dominant harmful effect.

It is preferred that the disorder is a disorder in which expression of a protein in the liver is defective, or which can be corrected by expression of a protein in the liver. It is particularly preferred that the defective expression in the liver of

the protein is responsible for any signs or symptoms of the genetic defect. Thus, it is preferred that the disorder is a disorder of a gene that is selectively expressed in the liver.

The gene product need not remain in the liver; the liver produces many polypeptides that are exported from the liver. For example, many blood components are synthesised in the liver. It is particularly preferred that the product is a blood component, for example a clotting factor. It is particularly preferred that the product is Factor IX or Factor VIII.

A further preferred product is insulin. This may be expressed in the liver by cells arising from the bone-marrow derived stem cell. The expression of insulin may be controlled temporally, for example by placing the sequence encoding insulin under the control of an inducible promoter, for example an inducible promoter under control of an exogenous small molecule, as discussed further below.

The product may be capable of promoting tissue repair, for example capable of promoting liver tissue repair/regeneration. For example, the product may be an antifibrotic, antiinflammatory, antiviral or anticancer agent.

Examples of agents that may act as antiviral agents include Interferons, particularly Interferon-α, Interferon-β and Interferon-γ; Tissue Necrosis Factor-α (TNF-α) and Interleukin 12 (IL12). Antiinflammatory agents include IL10, IL4 and IL5. Antifibrotic agents include Interferon-α, dHGF (Hepatocyte Growth Factor), TIMPS (Tissue Inhibitor of Metalloproteinases), and collagenase. Anticancer agents include agents that are capable of

inhibiting growth of cancerous cells, tumour growth or metastasis. These may include Interferon-α, TNF-α and IL12.

It will be appreciated that these terms include variants, fragments, derivatives or fusions of the naturally occuring molecules that retain at least the biological activity referred to above and as described in the references indicated below.

The following therapeutic genes may usefully be expressed within the liver:

1. HGF (hepatocyte growth factor). This stimulates liver regeneration may reverse cirrhosis.
2. IL-10 (interleukin-10). Interleukin-10 is anti-inflammatory within the liver; its expression has been shown to reduce the fibrosis within the liver of patients with hepatitis C who do not respond to interferon. It acts by reducing macrophage effector functions, reducing stellate cell activation and directing the immune response to a TH2 rather than TH1 Based response.
3. Interferon-alpha. This is used as an immune stimulant to treat hepatitis B and C and even in the absence of virus clearance reduces the incidence of liver cancer development in the cirrhotic liver.

Relevant references are as follows:

Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. International Interferon-alpha Hepatocellular Carcinoma Study Group. Lancet. 1998 May 23;351(9115):1535-9.

Benvegnu L, Chemello L, Noventa F, Fattovich G, Pontisso P, Alberti A. Retrospective analysis of the effect of interferon therapy on the clinical outcome of patients with viral cirrhosis. Cancer. 1998 Sep 1;83(5):901-9

Baffis V, Shrier I, Sherker AH, Szilagyi A. Use of interferon for prevention of hepatocellular carcinoma in cirrhotic patients with hepatitis B or hepatitis C virus infection. Ann Intern Med. 1999 Nov 2;131(9):696-701. Review

IL-10 reference: Nelson DR, Lauwers GY, Lau JY, Davis GL. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon
nonresponders. Gastroenterology. 2000 Apr;118(4):655-60

Thompson K, Maltby J, Fallowfield J, McAulay M, Millward-Sadler H, Sheron N.
Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. Hepatology. 1998 Dec;28(6):1597-606.

HGF references: Ueki T, Kaneda Y, Tsutsui H, Nakanishi K, Sawa Y, Morishita R, Matsumoto K, Nakamura T, Takahashi H, Okamoto E, Fujimoto J. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. Nat Med. 1999 Feb;5(2):226-30

Taniyama Y, Morishita R, Nakagami H, Moriguchi A, Sakonjo H, Shokei-Kim, Matsumoto K, Nakamura T, Higaki J, Ogihara T. Potential contribution of a novel antifibrotic factor, hepatocyte growth factor, to prevention of

myocardial fibrosis by angiotensin II blockade in cardiomyopathic hamsters. Circulation. 2000 Jul 11;102(2):246-52.

Sato M, Kakubari M, Kawamura M, Sugimoto J, Matsumoto K, Ishii T. The decrease in total collagen fibers in the liver by hepatocyte growth factor after formation of cirrhosis induced by thioacetamide. Biochem Pharmacol. 2000 Mar 15;59(6):681-90.

Fujimoto J. Gene therapy for liver cirrhosis. J Gastroenterol Hepatol. 2000 Mar;15 Suppl:D33-6. Review

Fujimoto J, Kaneda Y. Reversing liver cirrhosis: impact of gene therapy for liver cirrhosis. Gene Ther. 1999 Mar;6(3):305-6.

It is particularly preferred that the product is Interferon-α. As noted above, Interferon-α is considered to be effective in treating viral hepatitis and in preventing development of hepatic cancer, but its use is limited by side-effects and also expense.

The product may be expressed from any suitable genetic construct as is described below and introduced into the bone-marrow derived stem cell. Typically, the genetic construct which expresses the product comprises the coding sequence for the product (which may be an antisense molecule) operatively linked to a promoter which can express the transcribed polynucleotide (eg mRNA) molecule in the cell, which may be translated to synthesise the product polypeptide. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary

hosts, for example mammalian hosts, are typically provided in plasmid or viral vectors containing convenient restriction sites for insertion of the required DNA segment. Suitable promoters will be known to those skilled in the art, and may include promoters for ubiquitously expressed, for example housekeeping genes or for tissue-specific genes, depending upon where it is desired to express the product, as discussed further below.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

It is preferred that expression of the product is under the control of an inducible promoter. Preferably the inducible promoter is regulated by a drug-like molecule. It will be appreciated that it may be desirable to be able to regulate temporally expression of the product in the cell. Thus, it may be desirable that expression of the product is directly or indirectly (see below) under the control of a promoter that may be regulated, for example by the concentration of a small molecule that may be administered to the patient when it is desired to activate or repress (depending upon whether the small molecule, for example a drug-like molecule effects activation or repression of the said promoter) expression of the product. It will be appreciated that this may be of particular benefit if the expression construct is stable ie capable of expressing the product(in the presence of any necessary regulatory molecules) in the bone-marrow derived stem cell or cell derived therefrom for a period of at least one week, one, two, three, four, five, six, seven, eight months or one or more years. Thus, a preferred construct of the invention may comprise an inducible or regulatable promoter. Examples of regulatable promoters include those referred to in the following papers: Rivera *et al* (1999) *Proc Natl Acad Sci USA* **96(15)**, 8657-62 (control by rapamycin, an orally bioavailable drug, using two separate adenovirus or adeno-associated virus (AAV) vectors, one encoding an

inducible human growth hormone (hGH) target gene, and the other a bipartite rapamycin-regulated transcription factor); Magari *et al* (1997) *J Clin Invest* **100(11)**, 2865-72 (control by rapamycin); Bueler (1999) *Biol Chem* **380(6)**, 613-22 (review of adeno-associated viral vectors); Bohl *et al* (1998) *Blood* **92(5)**, 1512-7 (control by doxycycline in adeno-associated vector); Abruzzese *et al* (1996) *J Mol Med* **74(7)**, 379-92 (reviews induction factors e.g., hormones, growth factors, cytokines, cytostatics, irradiation, heat shock and associated responsive elements). Tetracycline – inducible vectors may also be used. These are activated by a relatively –non toxic antibiotic that has been shown to be useful for regulating expression in mammalian cell cultures. Also, steroid-based inducers may be useful especially since the steroid receptor complex enters the nucleus where the DNA vector must be segregated prior to transcription.

A further example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Rang & Will (2000) *Nucleic Acids Res* **28(5)**, 1120-1125 suggest that the tetracycline-responsive promoter may be repressed in the presence of Interferon-α Such a promoter may be useful in limiting the expression of Interferon-α so that it does not reach undesirable levels outside the liver. Alternatively, mutations to the promoter are suggested that may render it insensitive to Interferon-α. This may be useful in producing higher levels of Interferon-α than may be possible with the Interferon-α-sensitive promoter.

Where expression of the transduced gene is to be restricted to particular tissue(s), a construct using a tissue or cell specific regulatable element is used. Thus, the pluripotent stem cell may differentiate into several cell types but the gene of interest is only expressed in the tissue expressing transcription factors capable of transcribing the tissue specific regulatable element, for example is only expressed in liver. Expression may only occur once the bone-marrow derived stem cell has differentiated to the extent necessary for the relevant transcription factors to be expressed.

Expression of the product may be under the control of a tissue-specific promoter. The system may be further improved by regulating the expression at two levels, for example by using a tissue-specific promoter and a promoter controlled by an exogenous inducer/repressor, for example a small molecule inducer, as discussed above and known to those skilled in the art. Thus, one level of regulation may involve linking the product-encoding sequence to an inducible promoter whilst a further level of regulation entails using a tissue-specific promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the product from the inducible promoter).

The tissue-specific promoter may be a liver-specific promoter, for example the elongation factor α (EF1-α) promoter. Other liver-specific promoters include the albumin promoter, the transthyretin promoter (Quian *et al* (1995) *Mol Cell Biol* **15**, 1364-1376).

The cell-type specific promoter may be used directly to control the product gene, or to control the gene encoding the inducible transcription factor. Further

levels of control may also be used, as will be apparent to those skilled in the art.

A cholinergic specific promoter is described, for example in Naciff *et al* (1999) *J. Neurochem* **72**, 17-28, which describes the identification of a 6.4-kb DNA fragment from the mouse vesicular acetylcholine transporter (VAChT) gene, encompassing 633 bp of the 5'-flanking region of the mouse vesicular acetylcholine transporter (VAChT), the entire open reading frame of the VAChT gene, contained within the first intron of the ChAT gene, and sequences upstream of the start coding sequences of the ChAT gene, which is capable of directing cholinergic neuron-specific expression.

It is preferred that the bone-marrow derived stem cell further comprises a recombinant polynucleotide capable of expressing in a bone-marrow derived stem cell or cell derived therefrom a cytotoxic product (suicide product). The recombinant polynucleotide capable of expressing the cytotoxic product preferably is part of the same molecule as the recombinant polynucleotide encoding the therapeutic product, but this is not essential.

The capability for expressing a cytotoxic product provides the means for removing recombinant cells arising from the bone-marrow derived stem cell from a host. It is preferred that expression of the cytotoxic product is under the control of an inducible promoter (which is preferably a different inducible promoter to any inducible promoter controlling expression of the therapeutic product). It is further preferred that expression of the cytotoxic product is under the control of a non-cell-type specific promoter (so that cells may be removed regardless of how they have differentiated).

By cytotoxic product is included any gene product that is directly or indirectly toxic to the cell expressing the product. For example, the cytotoxic product may be an enzyme capable of activating a prodrug of a cytotoxic drug administered to a patient to the cytotoxic form. For example, the cytotoxic product may be a thymidine kinase. This is capable of activating gancyclovir to a cytotoxic agent, as known to those skilled in the art, thereby leading to death of the cell producing the cytotoxic product.

Expression of the cytotoxic product allows removal of the recombinant bone-marrow derived stem cells and differentiated cells derived from those cells. Thus, the invention provides means and methods for destruction of the exogenous gene-transduced stem cell after any therapeutic benefit has been obtained by the patient. Thus, the method may involve controlled inducible expression of a gene metabolising a non-toxic to a toxic compound, resulting in destruction of the stem cell. This safety system will allow destruction of the therapeutic stem cell after the desired therapeutic effect has been obtained.

As with other aspects of the invention, the operation of a chosen method of cell destruction may be tested in an animal model.

A further aspect of the invention provides a recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom a therapeutic product under control of a tissue specific promoter and an inducible promoter. The recombinant polynucleotide is preferably also suitable for expressing a cytotoxic product in a bone-marrow derived stem cell or cell derived therefrom.

A further aspect of the invention provides a recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom a therapeutic product and a cytotoxic product. Thus, the recombinant polynucleotide is bicistronic. It is preferred that expression of the therapeutic product is under the control of a tissue specific promoter and an inducible promoter. It is preferred that expression of the cytotoxic product is under the control of an inducible promoter (which is preferably a different inducible promoter to that controlling expression of the therapeutic product). It is further preferred that the cytotoxic product is under the control of a non-cell-type specific promoter.

It is preferred in relation to the cells and recombinant polynucleotide of the invention that the tissue-specific promoter is liver-specific, muscle-specific kidney or pancreas specific, or nerve cell-specific.

A further aspect of the invention provides a gene therapy construct comprising a recombinant polynucleotide of the invention. Preferably, the genetic construct is adapted for delivery to a human cell, still more preferably adapted for delivery to a human bone-marrow derived stem cell.

The construct may comprise a Moloney Leukaemia Virus (MLV) based retroviral vector, for example as discussed in Example 1. However, as noted in Example 1, such vectors may not be suitable if long-term expression may be required. The construct may alternatively comprise a lentiviral vector or adeno-associated vector (AAV), as discussed, for example, in Example 1 and Guenechea *et al* (2000) *Mol Ther* **1(6)**, 566-573. It is preferred that the construct comprises a lentiviral vector.

Means and methods of introducing a genetic construct into a cell *in vitro/ex vivo* or in an animal body are known in the art. For example, the constructs of the invention may be introduced into the cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the (dividing) cell. Targeted retroviruses are available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for gene therapy).

It will be appreciated that retroviral methods, such as those described below, may only be suitable when the cell is a dividing cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviral DNA constructs which encode said SNARE polypeptides may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a neo^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at –70°C. For the introduction of the retrovirus into the target cells, it is convenient to use directly retroviral supernatant to which 10 μg/ml Polybrene has been added.

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the

genome, for a longer time. An example of the latter approach includes liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653). Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle. It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into the bone-marrow derived cells. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144. Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia, lentivirus and parvovirus.

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cell types which over-express a cell surface protein for which antibodies are available. Thus, immunoliposomes may be useful for targeting to all or selected classes of bone-marrow derived stem cells bearing selected surface antigens, as discussed in Example 1. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-

phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be exposed to cells *in vitro/ex vivo.*

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the

cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the target cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage

through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown under conditions where they may express the polypeptide of the invention. Samples of cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant or cell can be detected using antibodies, as well known to those skilled in the art.

The genetic constructs of the invention can be prepared using methods well known in the art.

A further aspect of the invention provides a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct of the invention for use in medicine.

A further aspect of the invention provides the use of a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct of the invention or as defined above in the manufacture of a medicament for use in treating a patient in need of expression of a therapeutic product. It is preferred that the patient and the bone-marrow derived stem cell are human. Further preferences are as indicated above in relation to the cells, polynucleotides or constructs, as appropriate.

The patient may be in need of correction of a genetic disorder, or in need of tissue repair, including clearance of an infective agent, modification of a chronic inflammatory process, suppression of a fibrotic process, or control of tissue repair to control the risk of neoplasia. For example, the patient may have haemophilia or diabetes mellitus. The patient may have liver disease or be at risk of liver disease, for example at risk of cirrhosis or neoplasia. For example, the patient may have hepatitis A, B or C, or may have alcohol-induced cirrhosis of the liver. The patient may be at risk of cancer, for example as a consequence of tissue damage.

A further aspect of the invention provides a pharmaceutical preparation comprising a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct of the invention and a pharmaceutically acceptable carrier. The carrier(s) must be "acceptable" in the sense of being compatible with the bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

A further aspect of the invention provides a method of treating a patient in need of expression of a therapeutic product comprising the steps of (1) providing a

bone-marrow derived stem cell according to the invention (including a cell derived from the bone-marrow derived stem cell) or recombinant nucleic acid capable of expressing, in a bone-marrow derived stem cell or cell derived therefrom, a therapeutic product (for example a recombinant polynucleotide of the invention) (2) introducing the stem cell or recombinant nucleic acid into the patient.

It will be appreciated that the method includes introduction into a patient of cells derived from culturing recombinant stem cells outside the patient to a stage where they may no longer be pluripotent. It may be desirable to maintain cells in culture before administration to a patient in order to check that the cells are behaving normally, ie as expected, in particular to check that the cells are not malignant.

It is strongly preferred that the bone-marrow derived stem cells are derived from the patient being treated, but this not essential. It is preferred that the bone-marrow derived stem cell is provided by a method comprising the steps of (1) obtaining a bone-marrow derived stem cell from the patient, (2) introducing a recombinant nucleic acid as defined above, for example in relation to the first aspect of the invention, into the bone-marrow derived stem cell.

Methods of introducing the recombinant nucleic acid into the bone-marrow derived stem cell are indicated above. Methods of obtaining bone-marrow derived stem cells from patients, for example human patients are well known to those skilled in the art. The bone-marrow derived stem cells may be separated from other bone-marrow cells, or particular class(es) of stem cells may be separated from other class(es) of stem cell using techniques known to those

skilled in the art, for example as discussed in Example 1, but this is not considered to be essential. Immunosorting techniques, for example using fluorescence activated cell sorting, may be used.

It will be appreciated that the cells that are transfected or introduced into the patient may bone-marrow derived stem cells that may have started to differentiate or to be limited in relation to the cell types into which they are capable of differentiating. Such cells are included in the term "bone-marrow derived stem cell". The cells may have been cultured *in vitro* to such a state. By "bone-marrow derived stem cell" is included any cell derived from bone marrow that is capable of giving rise/differentiating into at least one non-hematopoietic cell type. Preferably the bone-marrow derived stem cell is capable of giving rise/differentiating into a hepatic cell, kidney cell, muscle cell or nerve cell, preferably a hepatic cell. It will be appreciated that a sample of bone marrow tissue is considered to comprise bone-marrow derived stem cells. Methods of obtaining and purifying bone-marrow derived stem cells are described, for example, in Lagasse *et al* (2000) *Nature Med* **6(11)**, 1229-1234 and in Example 1. Surface antigens characteristic of bone-marrow derived stem cells are described in Example 1, for example bone-marrow derived stem cells may be Sca-1^+ Lin^-.

The recombinant nucleic acid, for example recombinant polynucleotide or gene therapy construct of the invention, may be administered directly to the patient rather than to isolated bone-marrow derived stem cells, but may not be preferred, because a higher proportion of bone-marrow derived stem cells of the patient may be achieved by infecting cells *in vitro* rather than in the patient.

When the recombinant nucleic acid introduced into the bone-marrow derived cells (including *via* administration to a patient) comprises an inducible promoter, the method may comprise the step of administering to the patient a molecule that regulates expression from the inducible promoter, as discussed above.

It is preferred that the method comprises the step of administering to the patient a composition (mobilising composition) that is capable of promoting mobilisation of bone-marrow derived stem cells into the peripheral circulation. For example, the composition may comprise one or more cytokines, for example G-CSF (Granulocyte-colony stimulating factor).

The method may advantageously comprise the step of administering to the patient a composition (colonisation composition) that is capable of promoting the colonisation of a tissue by, and/or differentiation of, and/or replication of bone-marrow derived stem cells or cells derived therefrom. For example, when the patient is in need of generation of liver cells, it is preferred that the patient is administered one or more hepatic mitogenic or morphogenic growth factors. These may include the mitogenic peptides termed T3 (triiodothyronine), HGF (Hepatocyte Growth Factor) and KGF (Keratinocyte Growth Factor) [17, 18]. Thus, the colonisation composition may comprise T3, HGF and/or KGF.

A further aspect of the invention provides a kit of parts or composition comprising (1) (a) a bone-marrow derived stem cell of the invention or (b) a recombinant nucleic acid as defined above, for example recombinant polynucleotide or gene therapy vector of the invention, and an agent useful in

promoting transfection of bone-marrow derived stem cells, and (2) a mobilising composition as indicated above.

The kit or composition may further comprising a colonising composition as defined above and/or an inducing molecule as discussed above, for example tetracycline.

A further aspect of the invention provides the use of an inducing molecule as discussed above, colonising composition or mobilising composition in the manufacture of a medicament for treatment of a patient in need of expression of a therapeutic product.

The patient preferably has been, is, or will be administered an appropriate bone-marrow derived stem cell of the invention.

Preferably, the patient has liver disease. The patient may have or be at risk of infection with a hepatitis virus, for example Hepatitis A, B or C, still more preferably Hepatitis B or C.

It is preferred that the bone-marrow derived stem cell and patient are of the same species. It is particularly preferred that the bone-marrow derived stem cell and/or patient is human.

The aforementioned bone-marrow derived stem cells, compositions or constructs of the invention or a formulation thereof, or inducer molecule, may be administered by any conventional method including oral and parenteral (eg subcutaneous intramuscular) injection. Intraportal, intrasplenic or intrahepatic delivery, for example injection, are preferred. The treatment may consist of a

single dose or a plurality of doses over a period of time. It is preferred that the construct or bone-marrow derived step cells are administered by injection, preferably intramuscular, intraportal, intrasplenic or intrahepatic injection. It will be appreciated that an inducer, for example small molecule inducer as discussed above may preferably be administered orally.

Further delivery or targeting strategies may include the following. Ballistic compressed air driven DNA/protein coated nanoparticle penetration (i.e. BioRad device) of cells in culture or *in vivo* may be used. Plasmids for delivery should have cell-type specific promoters.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (for example, the construct of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for

injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient. It is considered that one administration of bone-marrow derived stem cells or construct as described above is sufficient, but it will be appreciated that further administrations may be required, for example if the recombinant cells or their product are immunogenic.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

It will be appreciated that the bone marrow derived stem cells, construct, compositions or inducer molecule can be delivered to a locus by any means appropriate for localised administration of a drug. For example, a solution of the said construct or composition can be injected directly to the site (for example, into the liver, or a site of tissue damage) or can be delivered by infusion using an infusion pump. The construct or composition, for example, also can be incorporated into an implantable device which when placed at the desired site, permits the construct or composition to be released into the surrounding locus.

The construct or composition, for example, may be administered via a hydrogel material. The hydrogel is non-inflammatory and biodegradable. Many such

materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10% to about 80% by weight ethylene oxide and from about 20% to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^{R}.

In this embodiment, the hydrogel is cooled to a liquid state and the construct or composition, for example, is admixed into the liquid to a concentration of about 1 mg nucleic acid per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the construct diffuses out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

The construct or composition, for example, can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as

ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the construct or composition. The construct or composition can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the compositions are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

The dose of the construct, for example, is dependent on the size of the construct and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of construct may be dependent on the size of the construct and the delivery vehicle/targeting method used but a suitable dose may be determined by the skilled person, for example making use of data from the animal and *in vitro* test systems indicated above.

The construct, for example, may be administered to the patient systemically for both therapeutic and prophylactic purposes. The construct, for example may be administered by any effective method, as described above, for example, parenterally (eg intravenously, subcutaneously, intramuscularly, intasplenically, intraportal or intrahepatic) or by oral, nasal or other means which permit the construct, for example, to access and circulate in the patient's bloodstream. Construct administered systemically preferably are given in addition to locally administered construct, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

As noted above, it is strongly preferred that the recombinant cells present in the patient may be destroyed when no longer needed, for example as a consequence of expression of a cytotoxic product in the cells.

The invention is now described by reference to the following, non-limiting figures and examples.

Figure 1: Defining conditions for bone marrow engraftment

Example 1: Haemopoietic stem cell therapy for the treatment of tissue damage, for example liver disease

Animal models are used for establishing the conditions that favour bone marrow cell engraftment and expansion in selected tissues, for example the liver.

1.1: Defining conditions for bone marrow self- engraftment

Bone marrow is considered to be useful in the healing of the liver after massive parenchymal loss. The creation of such a functional demand is likely to be a prerequisite for significant bone marrow engraftment. A murine model with marked bone marrow (see 2.1) whereby xenobiotic-induced severe acute liver injury can be created in a highly reproducible fashion from one animal to another is used. With the objective of attaining maximum self-engraftment of bone marrow into the damaged liver, rapid bone marrow mobilization into the peripheral circulation is caused with administered cytokines (e.g. G-CSF). The ability of a variety of hepatic mitogenic and morphogenic growth factors to

further facilitate the process is tested. The optimal conditions for bone marrow self-engraftment and transdifferentiation into hepatocytes/biliary epithelia are thereby defined, and this information may be exploited in the use of bone marrow in a therapeutic context.

1.2: Developing therapeutic scenarios

The use of bone marrow in therapy is attractive because bone marrow cells are easy to manipulate *ex-vivo*, unlike hepatocytes. Bone marrow cells may be used to deliver genes to the liver. This may be demonstrated using marker genes (EGFP/LacZ) under the control of liver-specific promoters (albumin). Examples of therapeutic genes include Factor IX (encoding a secreted protein), and interferon alpha (encoding a locally acting cytokine). The procedure may be tested in a model of chronic liver damage in the HBVsAg transgenic mouse (9,10).

2. Experimental design and methods

2.1 Cell Tracking

In order to track the fate of bone marrow cells in animal models, the experimental animals are lethally irradiated and transplanted with foreign (histologically recognisable) bone marrow cells at an early age. This can be done in a variety of ways, for example either transplanting male bone marrow into female mice and detecting the Y chromosome, or transplanting bone marrow from Enhanced Green Fluorescent Protein (EGFP) transgenic mice into wild type mice, or thirdly, transplanting bone marrow from ROSA-26 mice into wild type mice and histochemically or immunohistochemically detecting β-galactosidase activity. Recipient mice will be irradiated with 14 Gray gamma irradiation in two split doses 3-4 hours apart. Immediately following irradiation mice will receive murine cells from bone marrow donor

mice by intravenous injection as described previously (16). Mice will be housed under barrier conditions for at least 6 weeks prior to the detection of foreign bone marrow +/- procedures to encourage hepatic engraftment.

Examples of Detection system protocols

EGFP detection

This will be analysed by the UV exposure of whole tissue and thick sections and by the immunohistochemical detection of GFP.

Beta galactosidase transgene expression

Expression will be analysed in two ways: 1) histochemically by X-gal histochemistry as described by us previously (17) and, 2) immunohistochemically using standard procedures described by us (23)

2.2: Hepatic engraftment of bone marrow cells following liver damage

Bone marrow cell engraftment in the liver and all other major organs are monitored for up to a year after transplantation. In addition to normal recipients, murine models of liver damage are investigated. A specific hepatotoxin, paracetamol, will be administered to the mice to promote a functional demand for new parenchymal cells. At a minimum of 4 weeks after bone marrow transplantation, paracetamol at a dose of up to 350 mg/kg Body Weight (BW) is administered intraperitoneally on not more than two occasions separated by 7 days; each injection results in severe centrilobular necrosis affecting about 50% of the parenchyma. Secondly, β3-integrin knockout mice are used as recipients of bone marrow cells, since these mice suffer from chronic centrilobular hepatocyte destruction and thus, have a constant requirement for new hepatocytes (16). This model may indicate whether integrins are necessary for engraftment; engraftment will be defective if β3-integrin is required.

2.3 Modification of hepatic engraftment (functional colonization) of bone marrow cells by growth factors and cytokines

Haemopoietic stem cell engraftment in the liver might be reasonably be expected to be aided by mobilization of stem cells into the peripheral circulation. In the mouse, as in man, this is readily achieved by administering G-CSF. Furthermore, our previous studies (17,18) have identified some very powerful mitogenic peptides for liver (T3, HGF and KGF). Either singularly or in combinations these factors may improve the colonisation/differentiation of bone marrow cells in the liver and their subsequent expansion. These and other factors (or combinations thereof) may be tested for their ability to improve colonisation/differentiation, for example in order to identify useful combinations.

At a minimum of 12 hours after paracetamol administration, bone marrow cell mobilisation is achieved by up to 5 daily intraperitoneal injections of G-CSF at a dose of 10μg/kg BW. Following paracetamol and/or mobilization, growth factors (singularly or in combination) are administered immediately. All are administered for up to 3 weeks at weekly intervals: T3 is injected subcutaneously at a dose of up to 4mg/kg, HGF is injected intravenously at a dose of up to 1mg/kg and KGF is injected subcutaneously at a dose of up to 1mg/kg. The growth factor doses selected were the most effective in previous experiments designed to prime the liver for retroviral transduction; furthermore, we have noted a profound synergy of action when they have been administered in combination (17,18). In all cases, mice are killed at intervals for up to a year and liver, and all major organs are analysed for bone marrow cell engraftment. A time-course of engraftment, the percentages of mononucleate and binucleate hepatocytes from bone marrow and their zonal

distribution are plotted. Similar analysis of other tissues is performed. Similar experiments are performed in animal models of non-hepatic tissue damage.

Whole bone marrow may be used. Alternatively, haematopoietic stem cells (c-kit^{high}Thy^{low}Lin^{-}Sca-1^{+}) may be isolated from bone marrow cells by FACS. These may be used to determine if this particular sub-population has a pattern and efficiency of engraftment distinct from whole bone marrow; this may be useful in limiting the tissues in which engraftment takes place. For example it may be useful in limiting inappropriate transgene expression (e.g. interferon alpha in the brain). Mouse stem cells (Sca-1^{+} Lin^{-}) are purified using paramagnetic MACS microbeads (Miltenyi Biotech Ltd, Surrey, UK). The first step in this procedure is to enrich Sca-1^{+} cells using the Sca-1 multisort kit. The Sca-1^{+} Lin^{-} cells are then isolated from the Sca-1^{+} cells using a cocktail of FITC conjugated antibodies (CD2, CD3, CD4, CD5. CD8, NK 1.1, B220, Ter119, GR-1 and Mac-1); engraftment can be achieved with 50-1,000 of these cells (15). In some experiments, accessory cells such as T cells may be added to the transplant to improve levels of engraftment, or less highly purified inocula may be used in order to retain potential accessory cell effects.

2.4 Use of genetically modified bone marrow cells

We propose that bone marrow can be used as a vectoring system to deliver therapeutic genes to the liver (or other organ/tissue) in a clinical setting. Bone marrow can be transduced with foreign genes (19), and it is clinically more realistic to transfect autologous human bone marrow *ex-vivo* rather than harvest hepatocytes by partial hepatectomy (20) before transfecting them and returning them *via* the portal circulation (21). Genetically modified murine bone marrow

is transplanted to wild-type mice and to mice with liver damage, to examine whether genetic modification alters the normal pattern of engraftment in various organs. Liver is a target organ; brain, muscle and lung (amongst others) may also be affected.

Transgenic mice expressing the HBVsAg in the liver may be used as a test model. Expression of the surface antigen within the hepatocytes of transgenic mice leads to hepatocyte necrosis, chronic inflammation, hepatocyte regeneration and the development of hepatocellular carcinoma.

The interferon alpha gene is introduced into bone marrow cells because interferon alpha is an antiviral cytokine already used therapeutically. Interferon alpha used systemically leads to a 20%-30% probability of virus elimination and significantly reduces the risk of hepatocellular carcinoma development in cirrhotics, often in the absence of an anti-viral response (24, 25). However, systemic toxicity limits its use to the lower end of the dose-response curve. Thus, production of interferon endogenously by hepatocytes derived from bone marrow provides a much cheaper and more effective treatment. The effects of interferon alpha on the liver are monitored, particularly with respect to cell turnover, inflammation and tumour yield. Engraftment of the liver with bone marrow cells not containing the HBVsAg surface antigen may provide a sufficient stimulus for their colonization/differentiation and expansion. This selective pressure can be enhanced by performing splenic transfer experiments from donor mice that do not express the surface antigen but have been immunised with HBVsAg. The inflammatory hepatitis deletes HBVsAg-containing hepatocytes leading to repopulation of the liver by bone marrow-derived cells, a process that may be

augmented by growth factor(s) and mobilization of bone marrow into the peripheral circulation.

Reporter gene transfer may be used to assay initially for gene delivery to the liver of wild type mice by transduced stem cells and determine the length of time of gene expression. Our factor IX gene delivery approach is to treat haemophilic mice not expressing factor IX with vectors encoding the murine factor IX gene. The levels and length of time of gene expression may be determined by Elisa (or similar immunoassay or other detection method) of blood samples after treating animals by cauterisation to arrest bleeding. The levels of factor IX produced are compared with the minimum 1% of normal human levels required for a therapeutic effect. The factor IX approach also allows for transplantation of stem cells which may engraft in other areas of the body. The site of production of murine factor IX and high factor IX expression will not have delerious effects on the host. The use of murine factor IX circumvents an immune response to the transgene shown in our previous studies (22,23)). The preferred viral vectors are able to provide long-term gene expression over several months to years and are able to infect non-dividing cells, for example lentiviruses and adeno-associated viruses.

Genetic constructs

MLV based retroviral vectors generated by the TELCeB AF-7 packaging cell line (26) may be used; this produces high titre amphtropic pseudotyped lacZ encoding retroviruses (provided, for example, by our collaborator Y. Takeuchi). These vectors may be used for tracking stem cells, however, they may not guarantee long term gene expression due to promotor shutdown which is a common and not entirely understood feature of present retroviral vectors. To overcome this lentiviral and adeno-associated vectors (AAV) may be used.

Lentiviral vectors and AAV vectors have been shown to efficiently transduce stem cells in vivo (27-29). CMV promoter-driven lacZ and factor IX encoding AAV based vectors may be used. Preferred lentivirus vectors are based on HIV-1 and encode the GFP reporter gene. GFP are not only used to demonstrate the fate of the marked stem cells over time but the types of cells infected and the levels and longevity of expression.

Lentiviruses encoding the GFP reporter gene inserted downstream of the IFNα or factor IX genes are made as a single transcription unit using an internal ribosome entry site (IRES). This allows us to monitor successfully transduced cells and provide an accurate method to titre each of the new recombinant viral vectors. The elongation factor alpha (EF1-α) promoter may be used in each vector system, in place of the CMV promoter which may also be used. Although the CMV promoter has given long-term and high level factor IX gene expression in foetal mice, which we have already shown with an AAV vector (unpublished data), the (EF1-α) promoter may increase levels of gene expression. AAV vectors may be used with a inducible system combined with the CMV promoter and with the liver-specific EF1-α promoter. Although the tetracycline inducible system is commonly used for controlled gene expression, this system may not be suited to IFNα expression because IFNα autoregulates the tetracycline control system which, in turn, would cause uncontrolled IFNα expression (30). The recently described streptogramin gene regulation system may be used (31). This allows the study, reliably, of the effects of IFNα.

Tracking experiments, for example to establish the *in vivo* conditions that best promote bone marrow cell mobilisation and colonization of marked cells, may also be performed using stem cells marked with MLV based amphtropic pseudotyped lacZ encoding retroviruses (described above) for which highly sensitive PCR and immunohistochemical techniques for detection may be used.

Thus, the liver-specific elongation factor alpha (EF1-α) promoter may be used in each vector system described above. A tetracycline inducible control system (or other inducible control system) may be combined with the CMV promoter or with a tissue-specific promoter, for example the liver specific (EF1-α) promoter, for example in lentiviral or AAV vectors. This may be used to control expression of, for example, IFNα and factor IX therapeutic expression.

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CLAIMS

1. A bone-marrow derived stem cell comprising a recombinant nucleic acid capable of expressing, in the bone-marrow derived stem cell or cell derived therefrom, a therapeutic product.

2. The bone-marrow derived stem cell of claim 1 wherein the product is capable of correcting a genetic disorder.

3. The bone-marrow derived stem cell of claim 2 wherein the disorder is a disorder in which expression of a protein in the liver is defective.

4. The bone-marrow derived stem cell of any one of claims 1 to 3 wherein the product is a blood clotting factor.

5. The bone-marrow derived stem cell of any one of claims 1 to 3 wherein the product is insulin.

6. The bone-marrow derived stem cell of claim 1 wherein the product is capable of promoting tissue repair.

7. The bone-marrow derived stem cell of claim 1 or 6 wherein the product is an antiviral, antifibrotic, antiinflammatory or anticancer agent.

8. The bone-marrow derived stem cell of claim 1, 6 or 7 wherein the product is interferon-α or IL10.

9. The bone-marrow derived stem cell of any of the preceding claims wherein expression of the product is under the control of an inducible promoter.

10. The bone-marrow derived stem cell of claim 9 wherein the inducible promoter is regulated by a drug-like molecule.

11. The bone-marrow derived stem cell of any of the preceding claims wherein expression of the product is under the control of a tissue-specific promoter.

12. The bone-marrow derived stem cell of any of the preceding claims further comprising a recombinant polynucleotide capable of expressing in a bone-marrow derived stem cell or cell derived therefrom a cytotoxic product.

13. A recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom a therapeutic product under control of a tissue specific promoter and an inducible promoter.

14. A recombinant polynucleotide suitable for expressing in a bone-marrow derived stem cell or cell derived therefrom a therapeutic product and a cytotoxic product.

15. The bone-marrow derived stem cell or recombinant polynucleotide of claims 11 or 13 wherein the tissue-specific promoter is liver-specific, muscle-specific, pancreas-specific or nerve cell-specific.

16. A gene therapy construct comprising a recombinant polynucleotide as defined in claims 13, 14 or 15.

17. A gene therapy construct according to claim 16 wherein the construct comprises a Moloney Leukaemia Virus (MLV) based retroviral vector.

18. A gene therapy construct according to claim 16 wherein the construct comprises a lentiviral vector or adeno-associated vector (AAV).

19. A bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to any of the preceding claims for use in medicine.

20. Use of a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to any of the preceding claims in the manufacture of a medicament for use in treating a patient in need of expression of a therapeutic product.

21. The use of claim 20 wherein the patient is in need of tissue repair or correction of a genetic disorder.

22. A pharmaceutical preparation comprising a bone-marrow derived stem cell, recombinant polynucleotide or gene therapy construct according to any of claims 1 to 19 and a pharmaceutically acceptable carrier.

23. A method of treating a patient in need of expression of a therapeutic product comprising the steps of (1) providing a bone-marrow derived stem cell according to any one of claims 1 to 12, 15, or a recombinant nucleic acid as defined in any one of claims 1 to 15 (2) introducing the stem cell or recombinant nucleic acid into the patient.

24. The method of claim 23 wherein the bone-marrow derived stem cell is provided by a method comprising the steps of (1) obtaining a bone-marrow derived stem cell from the patient, (2) introducing a recombinant nucleic acid as defined in any one of claims 1 to 15 into the bone-marrow derived stem cell.

25. The method of claim 23 or 24 wherein the recombinant nucleic acid comprises an inducible promoter controlling expression of the therapeutic product and wherein the method comprises the step of administering to the patient a molecule that regulates expression from the inducible promoter.

26. The method of claim 23, 24 or 25 wherein the recombinant nucleic acid comprises an inducible promoter controlling expression of a cytotoxic product and wherein the method comprises the step of administering to the patient a molecule (cytotoxic inducer) that regulates expression from the inducible promoter controlling expression of the cytotoxic product.

27. The method of any one of claims 23 to 26 further comprising the step of administering to the patient a composition (mobilising composition) that is capable of promoting mobilisation of bone-marrow derived stem cells into the peripheral circulation.

28. The method of claim 27 wherein the composition comprises G-CSF (Granulocyte-colony stimulating factor).

29. The method of any one of claims 23 to 28 further comprising the step of administering to the patient a composition (colonisation composition) that is capable of promoting the colonisation of a tissue by, and/or differentiation of,

and/or replication of bone-marrow derived stem cells or cells derived therefrom.

30. The method of claim 29 wherein the tissue is liver.

31. The method of claim 29 or 30 wherein the colonisation composition comprises T3, HGF and/or KGF.

32. A kit of parts or composition comprising (1) (a) a bone-marrow derived stem cell according to any one of claims 1 to 12, 15 or (b) a recombinant polynucleotide or gene therapy vector according to any one of claims 13 to 18 and an agent useful in promoting transfection of bone-marrow derived stem cells, and (2) a mobilising composition as defined in claim 27 or 28.

33. The kit or composition of claim 31 further comprising a colonising composition as defined in any one of claims 29 to 31 and/or a molecule as defined in claim 25 and/or claim 26.

34. Use of a molecule as defined in claim 25 or 26, colonising composition as defined in any one of claims 29 to 31 or mobilising composition as defined in claim 27 or 28 in the manufacture of a medicament for treatment of a patient in need of expression of a therapeutic product.

35. The use of claim 34 wherein the patient has been, is, or will be administered a bone-marrow derived stem cell as defined in any one of claims 1 to 12, 15.

36. The use of claim 20, 21, 34 or 35 or method of any one of claims 23 to 31 wherein the patient has liver disease.

37. The use of claim 20, 21, 34 or 35 or method of any one of claims 23 to 31, or use or method of claim 36, wherein the patient has or is at risk of infection with a hepatitis virus.

38. The bone-marrow derived stem cell, use or method of any of the preceding claims wherein the bone-marrow derived stem cell and/or patient is human.

39. Any novel method of treatment, use, cell or nucleic acid as herein disclosed.

Figure 1

Defining conditions for bone marrow engraftment

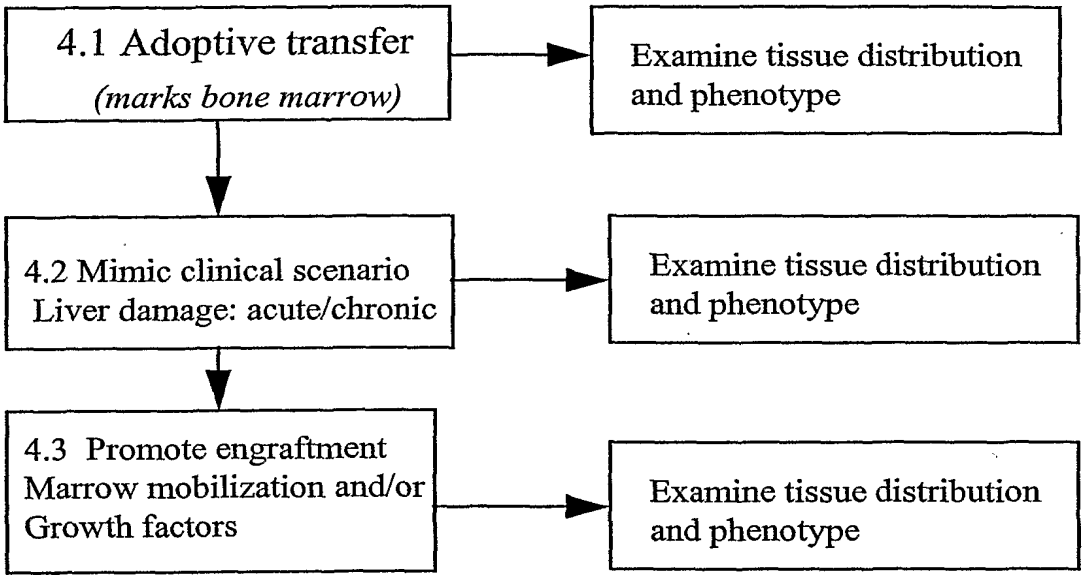
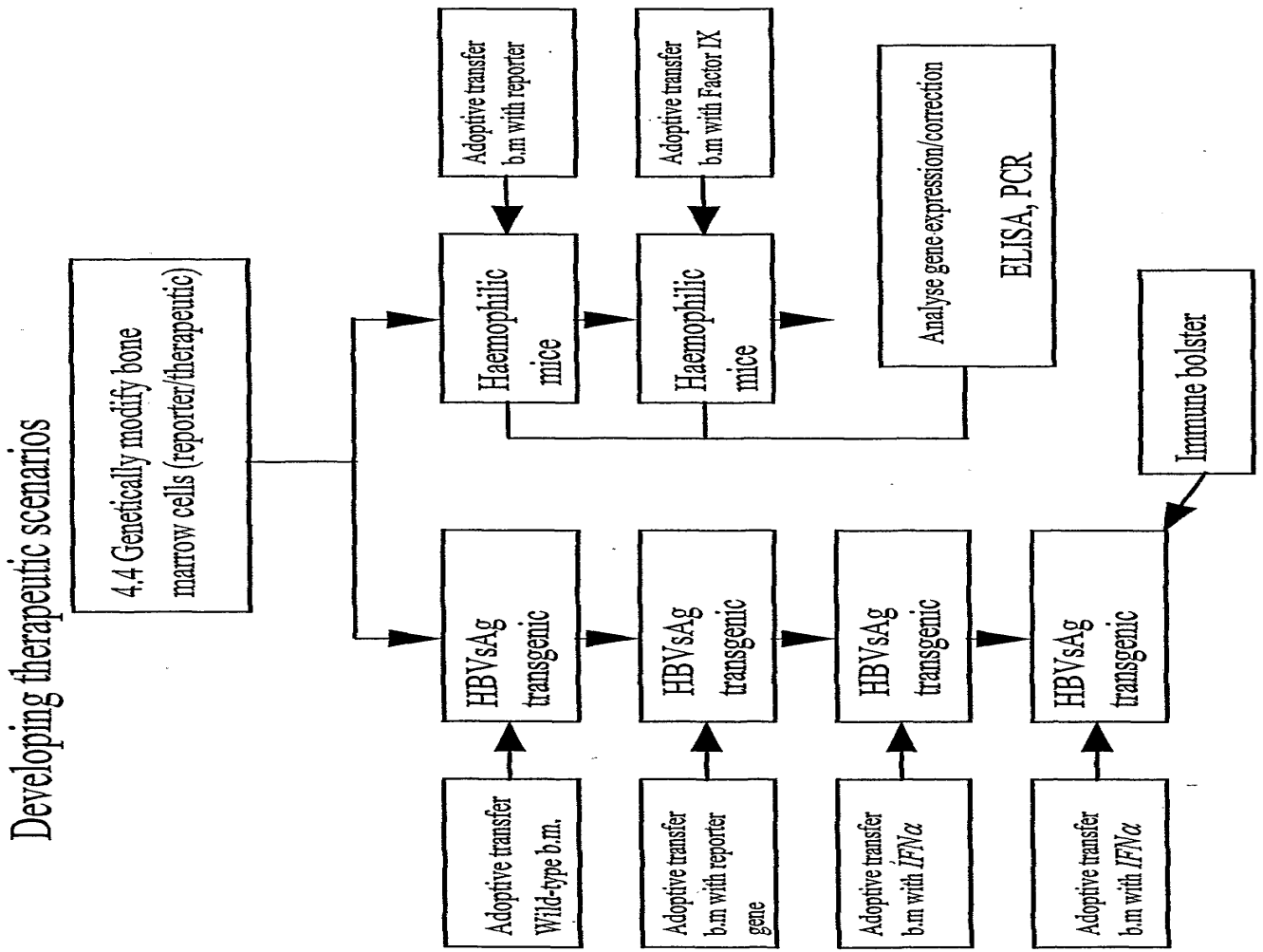


Figure 1


Developing therapeutic scenarios
4.4 Genetically modify bone marrow cells (reporter/therapeutic)
Adoptive transfer b.m with reporter
Adoptive transfer b.m with Factor IX
Haemophilic mice
Haemophilic mice
Analyse gene expression/correction
ELISA, PCR
Immune bolster
HBVsAg transgenic
HBVsAg transgenic
HBVsAg transgenic
HBVsAg transgenic
Adoptive transfer Wild-type b.m.
Adoptive transfer b.m with reporter gene
Adoptive transfer b.m with IFNα
Adoptive transfer b.m with IFNα

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